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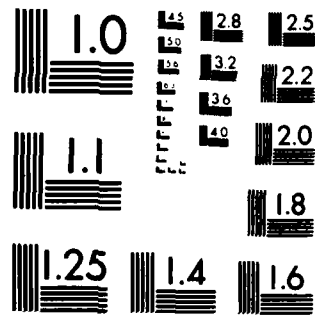
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The changes as a result of aging of the normal human erythrocyte, with special emphasis on the membrane proteins, have been investigated to determine whether they are related to the age-dependent macrophage uptake of the red cell. Al- terations in the metabolism can effect the arrangement of membrane proteins as evidenced by lectin agglutination of the red cells. Although serum proteins interact with the red cell, no alteration could be demonstrated in density-separated red cells.		

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Summary

The human erythrocyte has a well-defined life span of approximately 120 days. At the end of this time, the erythrocyte is removed from the vascular bed primarily by the macrophage of the spleen. The mechanism by which the red cell signals its age to the macrophage has been investigated. The possibility that the major membrane proteins such as the sialoglycoproteins are selectively removed or altered with increasing age has been explored. These proteins do not appear to be involved in the age-dependent signal for removal. The possibility that changes in the metabolic machinery of the red cell may be reflected on the outside surface has also been investigated by the use of lectin probes. Our results clearly show that surface changes can be modulated by the metabolism of the erythrocyte, although no direct relationship between age-dependent uptake and metabolism has as yet been established. We also investigated possible regulators of metabolism such as cAMP and have shown that they do effect posttranslational modification of membrane proteins by phosphorylation. Human red cells contain little or no adenyl cyclase, but we have shown that cAMP can enter the cell via the anion transport system. On the inside of the cell, it can effect changes at concentrations as low as 1×10^{-8} M. Thus, cAMP derived from other tissues may help regulate red cell membrane properties. Finally, we explored the possibility that plasma components may bind to the cell as a result of aging and signal the uptake. However, we found no differences in the amount of IgG bound by the red cell as a function of age.

For the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45CFR46.

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MEMBRANE PROTEINS OF THE ERYTHROCYTE AND THEIR ARRANGEMENT AS A FUNCTION
OF BOTH IN VITRO AND IN VIVO AGING

Final Report

Martin Morrison

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The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Introduction and Background

Role of Sialoglycoproteins of Erythrocyte Membrane in Signal for Aging

The most thoroughly studied aspect of membrane structure which has been related to aging has been the studies of the sialic acid and charge of the red cell as a function of age. There has, in fact, been considerable literature suggesting that the red cell loses sialic acid as a function of age (1-8), and this evidence was supported by other work which indicated that the overall negative charge of the red cell surface decreased as a function of age (9, 10). The charge on the red cell has been attributed primarily to the sialic acid content of the erythrocyte (9-11). Further, it was demonstrated that if red cells were treated with neuraminidase, which releases the terminal sialic acid residues of the sialoglycoproteins of the cell surface, the net negative charge of the red cell decreases, and the life span of the cells treated in this way also have been shown to decrease (4, 5, 7, 12, 13). As a matter of fact, when neuraminidase is injected into animals, the circulating red blood cells are affected, and the red blood cell survival time is reduced (4, 5, 7, 8, 12-15). Thus, there is a large body of evidence implicating the sialic acid as a determinant of age in the erythrocyte.

This evidence seems to correlate well with the recent observation by Morell and Ashwell (16) which shows that the survival of serum glycoproteins is a function of the sialic acid. Sialic acid is the terminal residue of the oligosaccharide units of these glycoproteins. When these terminal residues are removed, exposing the penultimate galactose unit, the glycoproteins are taken out of circulation. This system could be analogous to the uptake of the erythrocyte by the R-E system since in both cases, the terminal residue sialic acid is followed by penultimate galactose residue. In the case of the serum proteins, if the sialic acid is modified rather than removed, the uptake of the protein does not occur so that the signaling device is in fact the galactose residue.

The correlation between sialic acid content, charge and the signaling device for red cell uptake is not unequivocal. Evidence from a number of groups (17-20) have indicated that within experimental error, the sialic acid content of the old and the young cell does not vary significantly per gram membrane protein. The charge of the cells were also investigated by studying their mobility in electric field (17-20), and little experimental significant difference could be established between young and old cells.

Further evidence indicating that the sialic acid content or charge is not responsible for the signaling device of red cell uptake comes from the study of the sialoglycoproteins of the red cell membrane. In individuals lacking the antigenic determinant Ena, there is a large change in the sialic acid content and in the major sialoglycoproteins of the red cells. These cells contain approximately 45% of the sialic acid of the normal red cell (22-24), yet their survival is normal (21). Thus, the data is unclear on the role of sialic acid. There seems to be no doubt that sialic acid may play a role, but that it is not a simple relationship between sialic acid content and the aging signal. Sialic acid content of the cell is not the sole determinant for red cell uptake or aging.

Attempts have also been made to evaluate the role of terminal galactose residues of red cell glycoproteins. Jancik and Schauer treated rabbit red cells with the enzyme β -galactosidase. This enzyme can selectively remove β -linked galactose residues. Their treatment did not alter the half life of the treated cells, suggesting that the galactose residue is not a signal. However, these investigations never demonstrated that their treatment removed galactose from the cell under their conditions.

More recently, Bell, *et al.* (15) have studied the effect of treatment of red cells with galactose oxidase on life span of red cell. This enzyme can oxidize the C₆ carbon atom of the oligosaccharide terminal galactose or N-acetyl-galactosamine to an aldehyde. This modification of erythrocyte glycoprotein oligosaccharide did not alter the half life of dog or chicken cells, but did shorten the half life of the rabbit cells. The difference is probably due to the fact that these workers observed little oxidation attributable to galactose oxidase in the dog or chicken, while the oligosaccharide of the rabbit cells were markedly altered. The same group also removed the terminal sialic acid by first treating cells with neuraminidase which exposes the penultimate galactose unit. The cells were then treated with galactose oxidase. Although the neuraminidase greatly shortened the half life of the cells of all animals studied, the galactose oxidase treatment did not reverse this phenomenon in dog or rabbit and shortened the half life of the chicken cells.

Although these data may indicate that galactose terminal residues do play a role, particularly in the chicken cells, in the uptake of erythrocytes. The data is far from equivocal and indicates that terminal galactose itself is not a signal for the rabbit R-E cell to take the red cell out of circulation. However, alteration of the galactose may enhance uptake of the red cell by the R-E cell.

The aldehyde group generated in these experiments can react to form schiff base with amino-containing compounds. This reaction would alter the structure more dramatically than the conversion of an alcohol to an aldehyde suggests. This type of reaction is very likely and may account for the altered life span of the red cells observed in this study.

Interaction of Serum Proteins with Erythrocyte Plasma Membrane

The organization of membrane components depends not only upon the components of membrane but upon the interaction of these components with the proteins on the inside and on the outside of the cell. Several studies have indicated that the erythrocyte surface interacts with serum components. Autologous serum γ -globulin fractions, separable by cellulose phosphate chromatography, bind to erythrocytes both *in vitro* and *in vivo* (25) and serum albumin binds tenaciously to erythrocytes even after isotonic saline washing (26). With increasing times of storage under standard and experimental blood banking conditions, IgG, fibrinogen and albumin accumulation at the surface of the erythrocyte has been shown (27). Interaction of plasma lipoproteins and the apolipoproteins with erythrocytes has also recently been explored (29).

The serum protein component-erythrocyte surface interactions have a significant influence on the properties and function of the erythrocyte. Serum albumin affects the morphology of the erythrocyte (26-30), while autologous γ -globulin influences the resistance of human erythrocytes to hypotonic hemolysis and shearing forces (31), and in the dog, has been shown to be related to survival of the erythrocyte (32). Presence of autologous immunoglobulins on erythrocyte has been indicated to influence their phagocytosis by autologous macrophages, and there are significant variations in these interactions apparently depending on age or metabolic state of the erythrocyte (33). A specific enzymatic activity, Mg^{++} -ATPase, of human erythrocyte membranes has also been shown to be stimulated with very dilute solutions of low-density plasma lipoproteins (28).

Final Report

Our investigations during this contract have been along five lines.

I. Glycoproteins

There is a great deal of evidence which appears to implicate the glycoproteins of the red cell as being involved in the age-dependent uptake by the reticuloendothelial system. Thus, by implication, they are either directly or indirectly involved in the aging signal.

The human erythrocyte membrane glycoproteins may be divided into two groups, dependent upon the amounts of sialic acid present in the carbohydrate oligosaccharide of the protein. In the first group, with little or no sialic acid, are glycoproteins such as band 3, the major membrane protein of 90,000 molecular weight which is involved in anion transport. Its oligosaccharide chain is a single large oligosaccharide structure which terminates primarily in galactose residues and is linked to the protein through asparagine residues. The glucose transport protein, Band 4.5, also has a similar oligosaccharide chain. Both of these oligosaccharide chains can be degraded by endo- β -galactosidase which hydrolyzes Gal β 1-4GlcNAc linkages. Thus, these two important transport proteins in the membrane have N-acetyllactosamine repeating units (34).

The second group of glycoproteins are the sialoglycoproteins. We have evaluated the sialoglycoproteins in the human erythrocyte using new techniques developed in our laboratory. While previous investigations have considered the red cell to contain one or two sialoglycoproteins, our evidence using two-dimensional electrophoresis and discontinuous buffer systems have indicated clearly that there are at least 5 distinguishable sialoglycoproteins in the normal human erythrocyte. The intact normal human erythrocyte was labeled with lactoperoxidase which labeled most of the exposed membrane proteins. The sialoglycoproteins were partially purified from these cells, and the iodine-labeled sialoglycoprotein components were then separated by two-dimensional electrophoresis system. The polypeptides were first separated in a phosphate buffer then separated using a discontinuous buffer system. The major sialoglycoprotein, which we have shown contains 60% of the sialic acid of the red cell, is separated as PAS 1, which is a dimer; the monomer of this component runs with a molecular weight in the 40,000 region. A second component which we have previously distinguished and labeled PAS 2', runs in the same region as the monomer, but with a slightly higher molecular weight in the second dimension.

A third component labeled with lactoperoxidase is present as a monomer at 24,000 or dimer at approximately 48,000. Thus, lactoperoxidase labels three sialoglycoproteins.

We have also labeled the intact erythrocyte by the use of periodate-borohydride procedure. The periodate oxidizes the sialic acid generating an aldehyde on C7 and C8 carbon atoms. The aldehyde is then reduced with tritiated borohydride. This introduces the tritiated label into the sialoglycoproteins. These components are partially purified and separated in the same way as described above. The sialic acid labeling shows a number of other components present, but the component labeled 2' is not readily observed by this labeling procedure, as it contains little sialic acid. However, a component, PAS 2, which is not labeled by lactoperoxidase, is readily apparent. PAS 2 runs in the 35,000 molecular weight range and in contrast to 2', does not label with lactoperoxidase but is rich in sialic acid. It stains readily with the periodic acid Schiff-reagent. This component has been frequently confused with the monomer of PAS 1. Its dimer has a molecular weight of 70,000. Finally, there is a 27,000 molecular weight component which does not label with lactoperoxidase, but is labeled with periodate and borohydride.

Thus, there are clearly five components - PAS 1, which in the separation procedures employed is primarily a dimer but can be converted to a monomer; PAS 2 and 2'; PAS 3 and a fifth component which has a molecular weight of 27,000 and which we would call PAS 3'. Other components in addition to aggregates of the above sialoglycoproteins can be detected such as the 22,000 and 90,000 molecular weight bands, but have not been investigated extensively enough to determine whether any of these minor components are artifacts (35).

Neuraminidase treatment removes the sialic acid from all of these sialoglycoproteins. Thus, if only one of these components is involved in the aging signal, total sialic acid content would not be directly related to the uptake by the reticuloendothelial system. Our data clearly indicate that the major sialic acid-containing components PAS 1 and 3 which account for about 70% of the sialic acid of the red cell are not directly involved in the aging signal.

We have also investigated the number of galactose terminal residues in the erythrocyte as a function of age. The enzyme galactose oxidase will oxidize terminal galactose or galactosamine at the C6 carbon atom, producing an aldehyde. This aldehyde can be reduced with tritiated borohydride in order to incorporate a radioactive label into the membrane protein containing the galactose terminal oligosaccharide. We have worked out conditions that allow us to evaluate the terminal galactose on a quantitative basis, and have also investigated qualitatively the proteins containing the terminal residues. This provides a method of establishing whether the removal of the terminal sialic acid is taking place. Since there are as many as 20 residues of terminal sialic acid per mole of sialoglycoprotein, the removal of one or two sialic acid residues would only be a 5-10% change in sialic acid content. The increase in exposed galactose, however, would represent as much as 100-200% change as the sialoglycoproteins have few terminal galactose residues.

Although there is clearly a relationship between density and the age of the red cell, the correlation between these two parameters is far from perfect and relying on density alone may obscure the important aging signal. We, therefore, attempted to apply a second method for separation of the red cells, centrifugal elutriation. Centrifugal elutriation is a relatively new procedure for the separation of cells. With this method, the sedimentation of the cells in a centrifugal field is opposed by the counterflow of liquid and changes in either centrifugal field or flow rate can be used to fractionate a cell population. The separation achieved is based on size with small contribution due to shape and density. We found using the Beckman elutriator, an anomalous elution of cells. This elution resulted from a pulsed temperature drop in the centrifuge well, and caused a shift in modal cell size of the fractions. Because of this, the fractions have a larger size dispersion than they should theoretically have (36).

We eliminated this anomalous elutriation and separated red cells labeled by galactose oxidase-(^3H)-BII $_4$ procedure. No significant differences in specific activity and membrane components of young and old cells could be detected even when elutriation was confirmed with density separation techniques (37).

Homogeneity of protein 3 was also investigated and a variant of Band 3 was detected. Following proteolytic digestion of intact erythrocytes, Band 3 is altered to produce a 60,000 molecular weight fragment which remains associated with the membrane. However, in 5-6% of more than 500 individuals examined, digestion of the intact red cell generated two fragments - a normal 60,000 molecular weight and a 63,000 molecular weight fragment. Although the altered peptide is detected by proteolytic digestion of the external surface, the alteration appears to occur in that part of the molecule which is on the cytoplasmic surface. Proteolytic digestion at both membrane surfaces revealed that the normal and variant peptides had membrane-spanning segments of identical size, suggesting that the alteration is inside the cell. Thus, the 63,000 molecular weight fragment produced from 5-6% of the population has a larger molecule than normal protein 3, and this 3,000 increase in molecular weight is on the cytoplasmic surface of the membrane (38).

II. Surface Changes with Metabolism

We have probed the membrane surface employing lectin aggregation. For example, Con A and PHA, two lectins which have different carbohydrate receptors, react with largely distinct groups in the red cell membrane. PHA, for example, binds primarily to the major sialoglycoproteins while Con A binds primarily to protein 3. A quantitative assay for agglutination was developed and with this type of system, we have investigated the effects of the changes in the metabolic state of the cell. For example, agglutination by PHA seems to be dependent upon ATP levels. On the other hand, Con A agglutination is not directly influenced by the ATP levels alone (39).

We have attempted to correlate the functional activity of protein 3 with its activity as a receptor for concanavalin A. The concanavalin A agglutination of human erythrocytes is enhanced by adenosine. It varies with time of storage of the blood and is dependent on the concentration of adenosine in the medium. Adenine and/or inosine, which increase cellular ATP, do not substitute for adenosine in enhancing agglutination, and adenosine enhances agglutination of fresh erythrocytes with normal levels of ATP. Thus, it appears that cellular ATP levels are not directly involved in modulation of concanavalin A agglutination by adenosine. Trypsin, which hydrolyzes most of the exposed proteins of the cell surface but does not alter protein 3, enhances concanavalin A agglutination without altering the relative response of the cell to adenosine.

Glucose, as well as the glucose transport inhibitors maltose and cellobiose, inhibit agglutination. High concentrations of adenosine reverse the inhibition by glucose, and enhance agglutination in the presence of maltose and cellobiose.

Treatment of erythrocytes with 4,4'-diisothiocyanostilbene-2,2-disulfonic acid disodium salt, which selectively inhibits anion transport by protein 3, also inhibits adenosine-supported concanavalin A agglutination.

Treatment of erythrocytes with iodoacetate under conditions in which it selectively reacts with glyceraldehyde-3-phosphate dehydrogenase inhibits agglutination. Adenosine protects this dehydrogenase in erythrocytes from inactivation by iodoacetate, over the same concentration range in which it enhances agglutination (40).

III. Studies of Cytoskeletal Proteins

The red cell shape has as its primary determinants, the membrane bilayer and its underlying cytoskeleton. In order to investigate possible relationships of the cytoskeleton to age, the cytoskeleton proteins have been thoroughly investigated without unequivocal data implicating them in any genetic changes or aging changes.

This is in part due to the methods of study which have used the SDS electrophoresis as a primary tool for characterization. Genetic changes are more readily observed using methods based on charge differences.

For this purpose, a two-dimensional separation procedure was developed for the study of membrane proteins. In this procedure, the membrane was first solubilized in sodium dodecyl sulfate and separated first on the basis of charge by isoelectric focusing and then separated on the basis of size in the second dimension. By this procedure, over 90 discrete polypeptides could be detected in the pH range of 5 to 8. Special attention was given to the higher molecular weight components. For example, six components could be detected within the 90,000 to 100,000 molecular weight range of protein 3, the major membrane protein. A component with the same or very nearly the

same molecular weight as spectrin band 2 was detected. It is more basic than spectrin band 2, and both spectrin band 2 and the basic component are readily phosphorylated in the intact cell. However, the phosphorylation of band 2 is cAMP-independent while the phosphorylation of the basic component is enhanced by cAMP. In contrast to spectrin, the basic component is not extracted from the membrane with 0.1 mM EDTA, although dilute NaOH will remove it from the membrane. The Ca^{2+} -activated transferase of the erythrocyte cytoplasm will not crosslink this component. Calcium does, however, activate the conversion of this component to a lower molecular weight. This high molecular weight basic component has properties attributed to the component labeled 2.1 in Fairbanks' system of nomenclature (41).

IV. Studies of Serum Protein Binding to Erythrocytes

The fourth area of investigation has dealt with the binding of IgG to the red cells. The studies of the binding of IgG to erythrocytes can be subdivided into two groups. In one area of study, we have investigated the binding of IgG to the erythrocytes under low ionic strength conditions in a sucrose buffer. This fraction of the IgG which was earlier investigated by Najjar and his collaborators, is a fraction of IgG which was called erythrophilic IgG. This is a weak binding component which is easily washed from the cell in higher ionic strength. This type of interaction is very markedly affected by the removal of sialic acid groups with the amount of IgG decreasing as the sialic acid is removed from the erythrocyte. In a similar way, red cells which were treated with trypsin lose their ability to bind this erythrophilic IgG. The amount of IgG bound did not vary with the metabolic state. The binding of the erythrophilic IgG to young and old cells has been studied more extensively, and this binding also does not vary with the apparent in vivo age.

In a second study, we investigated the irreversibly bound IgG. For this study, red cells were extensively washed. The white cells and platelets were meticulously removed on cellulose columns. The extensively washed, carefully prepared red blood cells were found to have IgG by the fluorescent immunoassay procedures. The amount of IgG was very small and on the order of 100 molecules per cell. Distribution of this IgG when the red cells were lysed suggested that most of the IgG was bound to the membrane although 30-40% was in the supernatant fluid. A significant portion of the membrane-bound IgG can be eluted from the intact cells with low pH buffer (42).

V. Metabolic Regulators

The aging signal may not be determined by a single event, but may be the results of a sequence or series of events. We have, therefore, investigated one of the most likely mechanisms for interrelating metabolism with membrane protein; that is, the mechanism of phosphorylation of membrane proteins. Although previous workers have indicated that the sialoglycoproteins are not phosphorylated, we have found that these observations are a function of the experimental conditions. Under conditions more closely approximating the physiological conditions, we have observed the phosphorylation of the sialoglycoproteins. Our evidence indicates that the protein kinase will catalyze the phosphorylation of sialoglycoproteins to the extent of one mole of phosphate per mole of protein. The mechanism of control of phosphorylation of these proteins involves the cellular ATP. Although the

normal human erythrocyte has no adenylate cyclase and, therefore, cannot generate cAMP, we have investigated the transport of cAMP into the cell and its effect on phosphorylation of membrane proteins. We have found that there are two routes of cAMP uptake by the cells. A saturable uptake with a K_m of 3-5 mM and a V_{max} of 5 nM ml⁻¹ hr⁻¹ and a nonsaturable uptake with a rate constant of about 15 hr⁻¹. The saturable portion of the cAMP uptake can be blocked by treatment with DITS, suggesting that the cAMP entered the cell via the anion transport mechanism. When cells were suspended in a medium containing concentrations of cyclic AMP as low as 10⁻⁷ M, there was an effect on the phosphorylation of membrane proteins. Although cyclic AMP enhanced the amount of phosphate incorporated into the membrane employing inorganic phosphate as a substrate, this never amounted to more than 10% of the total phosphate incorporation. The most significant changes induced by cyclic AMP were on the distribution of the phosphate among the membrane proteins. Thus, it is possible that *in vivo*, the cAMP could be a mediator of changes in the membrane which could in turn result in alterations of the membrane protein which may be related to the aging of the cell (43).

Summary

Role of Glycoproteins

The oligosaccharide-containing glycoproteins of the normal erythrocytes can be subdivided into two groups based on the terminal carbohydrate residues: (a) the sialoglycoproteins, and (b) the galactose terminal glycoproteins. Group (a) is composed of four proteins which we identify as PAS 1, PAS 2, PAS 3 and PAS 2'. PAS 1 has also been labeled as glycophorin A and has a monomer molecular weight of 35,000 and contains between 60-80% of the sialic acid of the membrane. PAS 3 is closely related structurally, but not identical, with PAS 1 and contains about 10% of the cell sialic acid. PAS 2 contains between 8-10% of the total sialic acid of the red cell. PAS 2' is a minor component containing less than 1% of the total sialic acid of the cell.

The (b) group of glycoproteins is composed primarily of the major membrane protein 3 which transports anions and the glucose transport component, protein 4.5. The oligosaccharide chain of these proteins are linked to the protein through an asparagine residue. Both of these latter glycoprotein oligosaccharide chains can be degraded by the enzyme endo- β -galactosidase which hydrolyzes Gal β 1-4GlcNAc linkages. All of these glycoproteins on the outside of the red cell membrane are transmembrane proteins; that is, they are exposed on the outside of the cell and on the cytoplasmic surface of the plasma membrane.

Having established the types of glycoproteins on the erythrocyte membrane, we then evaluated whether they changed as a function of age of the cell. Red cells were separated into age-related groups, using density sedimentation and centrifugal elutriation procedures. An analysis of these glycoproteins for membrane protein showed no significant differences among these proteins. Thus, our preliminary conclusion is that the glycoproteins are not directly involved in the age-dependent signal for red cell uptake by the splenic macrophage.

In the course of our investigations, we detected a variant polypeptide of protein 3. The variant protein 3 has 3,000 higher molecular weight than normal protein 3. The change in the protein is on the portion of this trans-membrane protein that is exposed on the cytoplasmic side of the membrane, the amino terminal portion of the peptide. In a random population, about 5% of the population contained this variant peptide.

Role of Cytoskeleton

The possibility that the cytoskeletal components of the red cell membrane are important in aging of the red cell has been investigated with new two-dimensional electrophoresis separation procedures developed for these studies. Although these investigations did not provide unequivocal data implicating these proteins in any genetic or aging changes, these studies are being continued.

Role of Metabolic Regulation

The possibility that the outside surface of the red cell membrane was altered because of changes on the inside of the cell was investigated using lectin aggregation as a probe. The lectin Con A does not aggregate human cells very readily. However, in the presence of adenosine, this agglutination is markedly enhanced. This agglutination is not simply a function of cellular levels of ATP. The very selective anion transport inhibitor DIDS (4,4'-diisothiocyanostilbene-2,2-disulfonic acid) which reacts only with protein 3, inhibits adenosine-supported Con A agglutination. Thus, the adenosine effect appears to be specifically involved with protein 3.

The important metabolic regulator cAMP is transported into red cells by 2 routes - a saturable uptake with a K_m of 3-5 mM and a V_{max} of 5 mM ml^{-1} hr^{-1} and a nonsaturable uptake with a rate constant of about $1/ hr^{-1}$. Thus, red cells suspended in medium containing cAMP concentrations as low as 10^{-7} M have changes in the phosphorylation of membrane proteins. This raises the possibility that cAMP could be a mediator of red cell changes which may be related to the aging signal.

Binding of Plasma Components to Red Cells

Erythrophilic IgG is weakly bound to the red cell membrane and can readily be removed by washing with high-ionic strength buffers. The binding is decreased by removal of sialic acid from the cell. The binding of the erythrophilic IgG does not vary with the age of the cell.

In a second study, extensively washed cells were analyzed for irreversibly bound IgG using immunofluorescent assay procedures. The amount of IgG bound was about 100 molecules per cell. The possible role of immunoglobulins in the age-dependent red cell uptake is continuing.

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